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Full Length Research Paper

Effects of different vegetable oils formulations on temperature tolerance and storage duration of *Beauveria bassiana* conidia

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The aim of this work was to evaluate the effects of different vegetable oil formulations on the temperature tolerance and storage duration of *Beauveria bassiana* conidia. The germination ability of conidia mixed with eight vegetable oils including rapeseed, soy, sesame, corn, coconut, grape, olive and almond oils, were evaluated for the conidia temperature tolerance at 25, 30, 35, 40 and 45°C by spreading conidia over Sabouraud dextrose agar (SDA). The germination ability of conidia mixed with eight vegetable oils was evaluated after 1, 2 and 3 week storage at 25°C by spreading over SDA. Results show that there was significant difference between tolerance of conidia to different vegetable oils formulation so that the highest and the lowest germinations were established for variants with Sesame and Olive oils formulation respectively. The highest germination was proved at 25°C. The germination ability was reduced gradually by increasing the storage duration so that the percent germination reduction were 33, 44 and 52 for first, second and third week of conidia storage, respectively. The Sesame and Olive oils formulation had the highest and the lowest effects on conidial storage respectively.

Key words: Vegetable oils, *Beauveria bassiana*, temperature tolerance.

INTRODUCTION

The development of a suitable formulation is essential to the successful utilizetion of commercial mycoinsecticides (Daoust et al., 1983). For example, many formulations can affect the conidial viability resulting in a short shelf life (Moore and Prior, 1993). There is a need for careful assessment of the compatibility of formulation components with conidia prior to their use in formulations (Daoust et al., 1983). Therefore, one of the first steps in developing a mycoinsecticide formulation is to evaluate the effects of its components on conidial viability to select products compatible with fungal conidia. The development of fungal pathogen formulation depends on fungal strains, mass production ability and appropriate climate region (Butt et al., 2001). The most important factors limiting the use of fungi as an insecticide were solar ultraviolet radiation, temperature, humidity, and their ability on spreading on the surface (Staters et al., 1996). Formulating pathogens in oil enhances their infectivity compared to conventional water-based formulations (Agudelo and Falcon, 1983; Prior et al., 1988; Bateman et al., 1993). Views can differ on how long a mycoinsecticide shelf life is required, but estimates range from 3 to 18 months or even longer (Moore and Prior, 1993). The same authors say that it is desirable to maintain the viability to cover two cropping seasons, and long temperature storage is more for the convenience of the manufacturers than of the farmer, and thus should not be an obstacle. Knudsen et al. (1990) formulated the *Beauveria bassiana* mycelium in granules of sodium alginate with and without the addition of ground wheat. After five months of storage at room temperature, the fungi with most spore production came from the granules

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with wheat, with 2.45 × 10^8 conidia per granule. These, once placed on seedlings of wheat infested with *Schizaphis graminum* Rondani, caused the death of three to forty-four percent of aphis, against 0% in the control.

In general, temperature and moisture content, or the humidity of the storage atmosphere, are the major factors which influence conidial longevity (Hong et al., 1997). Hedgecock et al. (1995) studied the influence of moisture content on temperature tolerance and storage of *Metarhizium anisopliae* var. *acridum* in oil formulation and the results demonstrated that viability declined due to high temperatures and high moisture contents. Drying the conidia with silica gel greatly improved high temperature tolerance (McClatchie et al., 1994). The optimal moisture content for dried conidia storage was found to be 4 to 5% and a range of mineral oils proved satisfactory for dried conidia storage (Moore et al., 1996). Less moisture content than 4 to 5% may give better results but it is difficult to achieve. Suspoemulsions can be defined as heterogeneous formulations consisting of a stable dispersion of active ingredients in the form of solid particles and of fine globules in a continuous water phase combinations (GCPF, 1994). They are relatively new to the agricultural market and have a great potential for formulation and application of mycoinsecticides for pest control. They can be sprayed by very low volume/ controlled droplet application techniques still allow the use of conventional hydraulic sprayers and nozzles and water - the cheapest and most readily available carrier liquid for pesticides (Alves et al., 1998).

Little information is available about the effects of different commercially products available, which could be used in water-based formulations, on conidial temperature tolerance and shelf life. Hence, further studies are required. The aim of this investigation was: to evaluate the effects of different vegetable oils used as formulations on the temperature tolerance of *B. bassiana* conidia at 25, 30, 35, 40 and 45°C and on their germination ability after 1 to 3-week storage at 25°C.

MATERIALS AND METHODS

Formulations of *B. basiana* conidia

Conidia of *B. bassiana* isolate Iran 441C obtained from red palm weevil, *Rhynchophorus feroginus* Olivier (Coleoptera: Rhynchophoridae), in Iran, were used in this experiment because they were highly virulent against the Sawtoothed beetle, *Oryzaephilus surinamensis* L. (Coleoptera: Silvanidae), a model insect used in previous bioassays (Latifian et al., 2009).

Conidia of the fungal isolate were grown on Sabouraud-Dextrose-Agar (SDA) in a petri dishes (5 cm diameter, 1 cm depth), at 25 ± 0.5°C and under darkness for 10 days were harvested using a spatula and suspended in a total volume of 10 ml of 8 different formulations.

Conidia were formulated in different vegetable oil, including rapeseed, soy, sesame, corn, coconut, grape, olive and almond oils.

Conidia were mixed with vegetable oils and wetting/spreader agents prior to the addition of distilled water to obtain homogeneous suspensions. The stock formulation of each replicate was filtered using a sterilized muslin cloth then mixed using a Whirli Mixer (FSA Laboratory, U.K.) for 3 min. to break down conidial chains and to reduce clumping. All conidial formulations were calibrated at a concentration of 10^4 conidia ml^{-1} using an improved Neubauer's chamber. Then formulations after a 2 h rest were thoroughly agitated for 10 seconds using the Whirli Mixer. Aliquots of 0.1 ml from each formulation were then pipetted by an Eppendorf Research piston-stroke pipette and thinly spread over the SDA in Petri dishes. The plates were incubated at 25, 30, 35, 40 and 45 ± 0.5°C.

Conidial germination tests were carried out after 24 h. Conidia were examined at 400x magnification and germination was recorded when the germ tube was visible. All the conidia in each field of view were counted to obtain at least a total of 300 conidia in a range between 300 and 400, for each replicate (Moore et al., 1993).

The experiment had a factorial design with two main factors (formulations with eight levels and incubation temperature with 5 levels) and three replicates. Factorial analysis of variance (ANOVA) on conidial viability data was performed using the statistical package SPSS for Window (Norusis and SPSS, 1993).

Effects of oil formulations on duration storage of *B. bassiana* conidia

The germination ability of stored conidia from different formulations was assessed one day after formulation to obtain the initial germination level and at intervals of 1, 2 and 3 weeks. To facilitate the germination counting, 0.1 ml aliquots (containing approximately 10^4 conidia) from each stored conidial suspension were pipetted by an Eppendorf pipette and mixed with 5 ml of distilled water for the adjuvant oil treatments. For the pure dry conidia treatment, a small amount of conidia (approximately 0.007 g) was mixed with 5 ml of water plus 0.05% Tween 80 treatments (resulting in a suspension with approximately 1.43 × 10^6 conidia ml^{-1}). It was diluted 100 times to obtain approximately 1.43 × 10^4 conidia ml^{-1}.

All resultant diluted conidial formulations were then mixed using a Whirli Mixer for 3 min. to homogenize the suspensions. Finally, a new aliquot of 0.1 ml (containing approximately 10^4 conidia) was pipetted from each treatment and thinly spread over the SDA surface in a 5 cm diameter petri dish 1cm deep. The plates were incubated at 25 ± 0.5°C. Conidial viability tests were carried out after 24 h of incubation using the same methodology of the previous experiment (Moore et al., 1993).

This experiment had a factorial design with two main factors (formulations with eight levels and storage time with three levels) and three replicates. A two-way ANOVA on conidial viability data was performed using the same statistical package used in the previous experiment. The data were transformed to Arcsin $\sqrt{(\%/100)}$ to meet the requirements of ANOVA for a normal data distribution and homogeneity of variances.

RESULTS

Effects of different formulations on temperature tolerance of *B. basiana* conidia

There were significant differences between formulations (df = 7, 100, ms = 6151.6, P < 0.01) and between incubation temperature (df = 4, 100, ms = 10362.2, P < 0.01) on conidial viability. There was also a significant interaction between the main factors (df = 36, 100,

Table 1. Conidial viability of *B. bassiana* formulations 24 h after incubation at different temperature.

Formulation	Conidial viability ± SE (%)*				
	25°C	30°C	35°C	40°C	45°C
Rapeseed	89.3 ± 0.12^{B}	87.3 ± 0.11^{B}	77.7 ± 0.09^{C}	72.3 ± 0.09^{C}	61.5 ± 0.07^{D}
Soy	91 ± 0.17^{A}	89.67± 0.16^{B}	79.67 ± 0.14^{C}	74.47 ± 0.15^{C}	66.67 ± 0.12^{D}
Sesame	94.3 ± 0.14^{A}	94.1 ± 0.13^{A}	84.3 ± 0.12^{B}	79.6 ± 0.11^{C}	72.3 ± 0.09^{C}
Corn	77 ± 0.12^{C}	75.33± 0.09^{C}	58.67 ± 0.05^{E}	50.67 ± 0.04^{E}	39.3 ± 0.04^{G}
Coconut	78.2 ± 0.11^{C}	77 ± 0.1^{C}	65.8 ± 0.09^{D}	59.3 ± 0.04^{E}	48.3 ± 0.07^{F}
Grape	67 ± 0.11^{E}	64.7± 0.1^{E}	45.7 ± 0.09^{F}	38.3 ± 0.08^{G}	26.3 ± 0.02^{H}
Olive	57.6 ± 0.11^{F}	53.3± 0.1^{F}	7 ± 0.01^{J}	3 ± 0.01^{J}	1.4 ± 0.01^{J}
Almond	65.7 ± 0.11^{E}	63.3 ± 0.09^{E}	36.3 ± 0.03^{G}	25.7 ± 0.02^{H}	13.7 ± 0.01^{I}

*Standard error.

Table 2. Conidial viability of *B. bassiana* formulations after one, two and three weeks after storage.

Formulation	Conidial viability ± SE (%)*		
	One week	Two weeks	Three weeks
Rapeseed	88 ± 0.12^{B}	85 ± 0.11^{B}	85 ± 0.1^{B}
Soy	87.3 ± 0.12^{B}	86.33 ± 0.11^{B}	86.1 ± 0.11^{B}
Sesame	94.7 ± 0.14^{A}	91 ± 0.12^{A}	85.6 ± 0.11^{B}
Corn	76 ± 0.11^{C}	61 ± 0.09^{D}	43.7 ± 0.08^{E}
Coconut	78 ± 0.11^{C}	62 ± 0.09^{D}	52 ± 0.07^{E}
Grape	67 ± 0.09^{D}	46 ± 0.08^{F}	35.5 ± 0.05^{G}
Olive	57.4 ± 0.06^{E}	34 ± 0.04^{G}	24 ± 0.03^{H}
Almond	65 ± 0.08^{D}	45 ± 0.05^{F}	32 ± 0.04^{G}

*Standard error.

ms=472.9, P<0.01). These results are shown in Table 1.

The data were transformed to Arcsin $\sqrt{(\%/100)}$ to meet the requirements of ANOVA for a normal data distribution and homogeneity of variances. The results are presented untransformed in tables.

The experiments showed that conidial germination occurred between 5 to 45°C. Conidial germination on SDA after 24 h of incubation presented significant differences between formulations. Soil-based formulations haven't caused any negative effect on conidial germination. Soy and Sesame oils were significantly different from the other oils after 24 h, presenting the highest germinations. The germination of conidia was reduced by increasing the incubation temperature. The grape and almond oils gave the lowest conidial germination values after 24 h of incubation. The Sesame oil also gave the highest germination values after 24 h at 30°c. The soy and sesame oils gave the highest germination values after 24 h at 35, 40 and 45°c. The Olive oil gave the lowest conidial germination values after 24 h of incubation at 35, 40 and 45°c. Conidia formulated with all tested adjuvant oils gave mean values germination between 94.3 to 57.6, 89.67 to 53.3, 79.67 to 7, 79.6 to 3 and 72.3 to 1.4 for 25, 30, 35, 40 and 45°c respectively.

Effects of different formulations on medium-temperature storage of B. bassiana conidia

The viability of conidia stored in eight different formulations during one, two and three weeks was significantly affected by formulations (df = 7, 54, ms = 3822.1, P < 0.01), and storage period (df = 2, 54, ms = 5079.1, P < 0.01) (Table 2). There were also significant interactions between the main factors formulations x storage period (df = 16, 56, ms = 2669.04, P < 0.01), and between periods of storage and formulations over the first 3 weeks. Conidial viability within the same formulation significantly declined over time at different rates depending on the vegetable oils of the formulation. This effect was strongly marked between 2 and 3 weeks of storage.

The sesame oils also gave the highest germination values after one, two and three week storage. The olive oil gave the lowest germination values after one, two and three week storage. The rapeseed oil gave the same conidial germination values after one, two and three week

storage conidia formulated with all tested oils gave germination values between 94.7 to 57.4, 91 to 34 and 85.1 to 24 for one, two and three week respectively.

DISCUSSION

Compatible formulations with fungal conidia were selected in the first experiment. In practical temperatures, it was useful to carry out conidial viability tests after 24 h of incubation for the purpose of this experiment, because conidia were capable of recovery from adverse effects caused by some tested formulations and gave high germination levels, which could explain the significant interaction between formulations and incubation temperature. The wetting agent Tween 80 has been used in laboratory bioassays to facilitate suspension of hydrophobic conidia (Marques et al., 1981, Alves 1986, Prior et al., 1988). These results mean that the tested vegetable oils can be used to formulate *B. bassiana* conidia without permanent adverse effects on conidial viability. Non-ionic surfactants are the most common type of surface active agents, deriving their hydrophilic characteristics from nonionizable groups such as phenolic and alcoholic hydroxyls, carbonyl oxygens of esters and amides, ether oxygens, and analogous sulphur-containing configurations. Their nonionic nature is often advantageous in formulations because of their lack of reactivity with ions present in hard water (for example, calcium, magnesium, or ferric ions) and their chemical compatibility with many other chemicals (Field and Dastgheib, 1996). Cationic surfactants ionise in water such that the hydrophilic group becomes positively charged. Primary, secondary, tertiary, and quaternary amino groups and ammonium cations are the most common types of cations formed by these surfactants (Field and Dastgheib, 1996). The two cationic wetter/ spreaders possibly ionised in water and formed amino groups and ammonium cations, which were toxic to the fungal conidia. It is possible that these types of products are more appropriate to be added to chemical pesticides and not to biological pesticides.

In the experiment on temperature tolerance of conidia, viability was better maintained at 25°C than 30°C for all tested formulants. The conidial viability within the same formulation significantly declined with increase of storage period. Practical requirements for field applications are minimal loss of viability after at least three weeks of storage at 30°C. If at least three weeks of storage are required, cooled storage will be necessary (Moore et al., 1996). In this work, conidia formulated in all types of oils retained > 24% viability after three weeks of storage at 25°C. These results explain the significant interaction between temperature and formulants and between formulants and storage period.

Stathers et al. (1993) obtained no low viability when conidia were stored in Codacide, peanut oil and Shellsol for more than one week at 25°C. This was possibly due to the fact that conidia with high moisture content were scraped directly from agar slopes without drying. These results confirm that oil formulation conidia greatly improved high temperature tolerance and conidia need to be placed in oil (McClatchie et al., 1994).

The reason why the conidial viability declined more in the vegetable oils after three week stored at 25°C, could be explained by the presence of emulsifiers in their composition as mentioned above (Field and Dastgheib, 1996). The emulsifiers did not cause any pronounced adverse effect during the first one week, but after that, the conidial viability could be affected at different rates, depending on the composition of the formulations. This could explain the significant interaction between formulations and storage period and between formulations, temperature and time of storage. Conidial virulence was not tested in this work, but results from a study carried out by Moore et al. (1995), where the conidial viability declined over 37 months in storage, showed that the virulence of the formulations was not reduced after 30 months.

There is a little published information available about the formulation of entomopathogenic fungi because the technology is still held as an industrial secret. However, it is known that it is a mixture of several compatible products that include an active ingredient, typically conidia, a thinner and or a disperser, a wetting agent and an adherent (Latgé and Moletta, 1988). More recently, the production of these fungi have been done in the form of dry mycelium, since this could best resist the adverse condition until it comes in contact with the agent.

Results from the present work showed that vegetable oils fungal formulations can be used to formulate and store conidia for medium-temperature and probably for long-temperature under cooled conditions. In addition, they can also be sprayed with the existing delivery systems and used in broad scale agriculture where water-based formulations are predominant.

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